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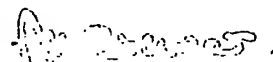
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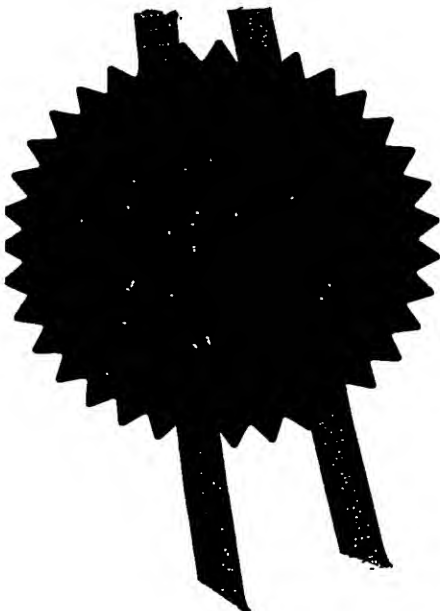
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United Kingdom

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation 07409436004
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4. Title of the invention THERAPEUTICALLY USEFUL MOLECULES

5. Name of your agent (*if you have one*)

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THERAPEUTICALLY USEFUL MOLECULES

The present invention relates to therapeutically useful molecules, in particular to T cell receptors (TCRs) which may be introduced into a patient's own T cells in order to direct the T cells to kill cancer cells within the patient, particularly cancer cells which express the Wilms Tumour antigen-1 (WT1).

The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge. All of the documents referred to in this specification are hereby incorporated by reference.

There is evidence that anti-tumour cytotoxic T lymphocytes (CTL) play an important role *in vivo*. Tumour reactive CTL have been shown to mediate tumour regression in animal models (Kast *et al* (1989) *Cell* 59, 603-614) and in man (Kawakami *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91, 6458-6462; Dudley (2002) *Science* 298, 850-854). As with all types of anti-tumour therapy, a problem that needs to be overcome is that the therapy must destroy or inactivate the target tumour cells to a useful extent but that the therapy must not destroy or inactivate non-tumour cells to a deleterious extent. In other words, it is desirable if the therapy is selective for tumour cells to a beneficial extent.

25

Much of the current work on immunotherapy of cancer makes use of the fact that certain tumours express polypeptides which are not expressed in the equivalent non-tumour tissue, or makes use of the fact that the tumour expresses a mutant form of a polypeptide which is not expressed in the non-tumour tissue. However, it is not always possible to identify polypeptides

30

in a tumour which fall into this category, and so other target polypeptides which can form the basis of an immunotherapeutic approach have been identified.

- 5 In adults, expression of WT1, an embryonic transcription factor, has been observed in renal podocytes, in the testis, in the ovary, in breast myoepithelial cells and in some CD34⁺ stem cells in the bone marrow. Aberrant expression was observed in breast cancer, ovarian cancer, melanoma, lung cancer, colon cancer, thyroid cancer, head and neck cancer, glioblastoma, sarcoma and leukaemia including CML and AML (see, for
10 example, Menssen *et al* (1995) *Leukaemia* 9, 1060-1067; Inoue *et al* (1997) *Blood* 89, 1405-1412; Inoue *et al* (1996) *Blood* 88, 2267-2278; Inoue *et al* (1998) *Blood* 91, 2969-2976; Menssen *et al* (1997) *Int. J. Cancer* 70, 518-523; Menssen *et al* (1995) *Leukemia* 9, 1060-1067; Ogawa *et al* (1998) *Transplant* 21, 527-527; Rodeck *et al* (1994) *Int. J. Cancer* 59, 78-82;
15 Silberstein *et al* (1997) *Proc. Natl. Acad. Sci. USA* 94, 8132-8137; Tamaki *et al* (1996) *Blood* 88, 4396-4398; Viel *et al* (1994) *Int. J. Cancer* 57, 515-521; Menssen (2000) *J. Cancer Res. Clin. Oncol.* 126, 226-232; Miyoshi (2002) *Clin. Cancer Res.* 8, 1167-1171; Oji (1999) *Jpn J. Cancer Res.* 90, 194-204; Oji (2003) *Cancer Sci.* 94, 523-529; Oji *et al* (2003) *Cancer Sci.*
20 94, 606-611; Oji *et al* (2003) *Cancer Sci.* 94, 712-717; and Ueda (2003) *Cancer Sci.* 94, 271-276.

As described in our patent application WO00/26249, using an
25 unconventional approach employing allo-MHC-restricted CTL, we identified peptide epitopes in the WT1 polypeptide which may be presented by HLA-A2 class I molecules and displayed on the surface of tumour cells expressing these proteins endogenously. HLA-A2 negative responder individuals were used as a source of CTL specific for peptides presented by
30 HLA-A2 class I molecule, and this approach allows identification of HLA-

A2 presented peptides independent of possible tolerance of autologous CTL.

One of the peptide epitopes disclosed in WO00/26249 is RMFPNAPYL (which we have also termed pWT126), and we have previously described a CTL which is able to: kill HLA-A2-positive targets coated with the WT1-derived peptide pWT126 (Gao *et al* (2000) *Blood* 95, 2198-2203); kill fresh HLA-A2-positive leukaemia cells expressing WT1 (Gao *et al* (2000) *Blood* 95, 2198-2203); kill HLA-A2-positive leukemia CFU progenitor cells (Gao *et al* (2000) *Blood* 95, 2198-2203; Bellantuono *et al* (2002) *Blood* 100, 3835-3837); kill HLA-A2-positive leukaemia LTC-IC stem cells (Bellantuono *et al* (2002) *Blood* 100, 3835-3837); kill HLA-A2-positive NOD/SCID leukaemia initiating cells (Gao *et al* (2003) *Transplantation* 75, 1429-1436); and do not kill normal HLA-A2-positive NOD/SCID engrafting hematopoietic stem cells (Gao *et al* (2003) *Transplantation* 75, 1429-1436). However, none of these publications give molecular information concerning the TCR present in the CTL, and the particular CTL line mentioned in the publications has not been made available to the public in any way and so the structure of the TCR is unknown and could not be derived by the skilled person (since the CTL line was not publicly available).

The present inventors have now cloned a TCR that is specific to RMFPNAPYL, a peptide of WT1 which is presented by HLA-A2 class I molecules, and have shown that introducing the TCR into either CD4-positive or CD8-positive T cells confers on the engineered T cells the ability to kill cancer cells which express WT1 endogenously. In addition, the inventors have defined the molecular structure of the TCR, identified the complementarity determining regions (CDRs), and describe how to make

recombinant TCRs which are believed to retain the same specificity of the parent molecule.

The TCRs may usefully be introduced into a T cell derived from a patient (preferably an HLA-A2-positive patient) suffering from a malignancy (where the patient's tumour cells express WT1), and the engineered T cell introduced into the patient in order to combat the malignancy. In particular, it is proposed to take T cells from patients with breast cancer, colon cancer, lung cancer, other solid cancers or leukaemia, transduce them *in vitro* with a retroviral vector containing the TCR genes, and re-infuse the transduced T cells into the patients. The credibility of this approach is confirmed by the demonstration in the Examples that the WT1-specific TCR genes can be transferred into human T cells, that the genes give rise to TCR expression on the surface of recipient T cells, that the recipient T cells can kill HLA-A2-positive target cells coated with the pWT126 peptide and HLA-A2-positive tumour cells expressing WT1 endogenously.

The general structure of T cell receptors (TCRs), their domain structure and the organisation of genes that encode them is well known, for example see Chapter 11 in Immunology, second edition (1994), by Janis Kuby, W H Freeman & Co, New York, USA, and Garcia *et al* (1999) *Ann. Rev. Immunol.* 17, 369-397. One common class of natural TCRs is the $\alpha\beta$ class in which the TCRs are made up of a separate alpha chain and a separate beta chain which form a heterodimer which is T cell membrane associated. Each alpha and beta chain is made up of regions which, in order from the N terminus to the C terminus are a leader sequence, a variable region, a constant region, a connecting sequence, a transmembrane region and a cytoplasmic tail region (see Figure 14 for a graphical representation of $\alpha\beta$ TCR structure). The variable region of the alpha chain is called the $V\alpha$ region and the variable region of the beta chain is called the $V\beta$ region.

Similarly, the constant region of the alpha chain is called the C α region and the constant region of the beta chain is called the C β region. The job of the $\alpha\beta$ TCR is to recognise and bind to a peptide presented in a HLA molecule of a cell in the body. Generally speaking, the TCR cannot recognise and bind the peptide unless it is presented by a particular HLA molecule, and the TCR cannot recognise a HLA molecule unless it is presenting the specific peptide. T cells harboring a specific TCR will target cells which are presenting a specific peptide in a particular HLA molecule on a cell (ie a peptide-HLA complex), and this is the main principle of T cell-based immunity.

The peptide-HLA complex is recognised by the combined V regions of the alpha and beta chains of the TCR. In particular, it is the complementarity determining regions (CDRs) of the V regions which mediate recognition of the peptide-HLA complex. The V region of the alpha and beta chains of the natural TCR are made up of, in order in an N-terminal to C-terminal direction, FR1, CDR1, FR2, CDR2, FR3 and CDR3, where FR stands for "framework region" and CDR stands for "complementarity determining region". The FRs and CDRs of the alpha and beta chains are different.

From the predicted amino acid sequences of the alpha and beta chains of the TCR cloned as mentioned above, the inventors have determined the FRs and CDRs of the alpha and beta chains (see Figures 2 and 4). With the knowledge of the CDR sequences, it is possible to produce chimaeric TCRs in which the CDRs are grafted onto framework regions with which the CDRs are not naturally associated, and it is also possible to produce single chain TCR molecules, and in both cases the molecules retain substantially the same binding affinity for the peptide-HLA complex as the parent molecule, as is described in more detail below.

A first aspect of the invention provides a T cell receptor molecule (TCR) containing an alpha chain portion and a beta chain portion wherein the alpha chain portion contains three complementarity determining regions (CDRs):

5 CDR1 α : SSYSPS

CDR2 α : YTSAATL

CDR3 α : VVSPFSGGGADGLT

and the beta chain portion contains three complementarity determining regions (CDRs):

10 CDR1 β : DFQATT

CDR2 β : SNEGSKA

CDR3 β : SARDGGEG, or wherein up to three amino acid residues in one or more of the CDRs are replaced by another amino acid residue.

15 The standard IUPAC one letter amino acid code is used throughout the specification. For the avoidance of doubt, a reference to a "particular" or "given" CDR means any CDR with the amino acid sequence given above or wherein up to three amino acids have been replaced by another amino acid residue.

20 By "TCR molecule" we include any molecule which contains the given CDRs and also contains FRs suitably situated within the molecule so that the CDRs form a recognition site (combining site) which is able to bind to HLA-A2 presenting the peptide RMFPNAPYL (ie a HLA-
25 A2/RMFPNAPYL complex).

It is particularly preferred if the TCR molecules contain the precise CDR amino acid sequences as given above and in Figures 2 and 4. Where a variant to this precise sequence is present, it preferably varies by one or two
30 or three (preferably one or two) amino acids in one or two or three or four

or five or all six CDRs. Typically, in these variants, the amino acids which are replaced are replaced with conservative amino acids. By conservative amino acids we include the groupings: G, A; S, A, T; F, Y, W; D, E; N, Q; and I, L, V.

5

A method for making and selecting TCR molecules which have CDRs which vary from the precise CDR sequences given in Figures 2 and 4 is given below.

- 10 The amino acid sequences, including V regions (and therefore FRs), of numerous TCR alpha chains and TCR beta chains are well known in the art, some of which are described in the IMGT (Immunogenetics) database at <http://imgt.cines.fr>. See also Lefranc (2003) *Dev. Comp. Immunol.* 27, 55-77. The structural basis of T cell recognition is reviewed in Garcia et al
- 15 (1999) *Ann. Rev. Immunol.* 17, 369-397, and the information contained therein may be used to design and synthesise CDR-grafted TCRs. Preferably, the FRs into which the particular CDRs are grafted are FRs of human TCR alpha or beta chains. Conveniently, the alpha chain CDRs are grafted into alpha chain FRs, and beta chain CDRs are grafted to beta chain
- 20 FRs. Typically, the three CDRs in the alpha chain and the three CDRs in the beta chain replace, in order, CDRs in other human alpha and beta chains, respectively. See Lefranc (2003) *Dev. Comp. Immunol.* 27, 55-77.

Typically, T cells expressing the TCR molecule recognise the HLA-A2

25 presenting peptide RMFPNAPYL with substantially the same avidity as the TCR molecule which consists of the alpha and beta chains as described in Figures 2 and 4. This can be measured by retroviral-mediated transfer of the TCR into T cells followed by peptide titration experiments with the TCR-transduced T cells as outlined, for example, in Gao *et al* (2000) *Blood*

30 95, 2198-2203.

The TCR molecule preferably contains an alpha chain portion containing, in N-terminal to C-terminal order, FR1 α -CDR1 α -FR2 α -CDR2 α -FR3 α -CDR3 α , and a beta chain portion containing, in N-terminal to C-terminal order, FR1 β -CDR1 β -FR2 β -CDR2 β -FR3 β -CDR3 β as shown in Figures 2 and 4, respectively. Typically, the TCR molecule contains the V region of both the alpha chain and the beta chain of the TCR polypeptide chains shown in Figures 2 and 4.

10 In a preferred embodiment, the alpha chain portion and the beta chain portion are present on different polypeptide chains. Typically, the TCR molecule contains an alpha chain which contains the V region and the C region of the polypeptide chain shown in Figure 2, and also contains a beta chain which contains the V region and C region of the polypeptide chain shown in Figure 4. Preferably, the TCR molecule consists of a molecule containing the complete alpha chain shown in Figure 2 and the complete beta chain shown in Figure 4. Typically, however, the leader sequence is cleaved off the mature alpha chain and beta chain.

20 In a further embodiment, the alpha chain portion and the beta chain portion of the TCR molecule are present in the same polypeptide chain. Single chain TCR molecules are described in Chung *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91, 12654-12658, and the principles described therein may readily be applied to the production of single chain TCR molecules which contain the specified CDRs. Typically, the single chain TCR molecules contain the V α , V β and C β domains fused in the same polypeptide chain, and typically in that order (from N-terminus to C-terminus). For expression of a single chain TCR it is useful to provide a construct encoding the constant domain of the TCR alpha chain.

An additional strategy is described in Boulter *et al* (2003) *Protein Eng.* 16, 707-711 in which a new disulphide bond is introduced between a threonine in the constant region of the alpha chain and a serine in the constant region of the beta chain (by replacing these residues with a cysteine. The
5 disulphide bond in the TCR connecting peptide may be removed or may remain in place.

The two-chain TCR molecules of the invention (ie ones which contain the
10 alpha and beta chains whose amino acid sequence is given in Figures 2 and 4) or chimaeric TCRs which contain the specific CDRs as described above may be used to introduce to create antigen-specific CTL as described in more detail below (by using polynucleotides that encode the relevant chains). Similarly, the single chain TCRs may also be used for this purpose,
15 and have the advantage that they do not pair with endogenous TCRs. Single chain TCRs may also be used as soluble constructs in a way similar to antibodies. In this case, the single chain constructs do not contain a transmembrane region (see Chung *et al supra* and Boulter *et al supra*).

20 A second aspect of the invention provides a polynucleotide encoding the alpha chain portion as defined in the first aspect of the invention. A third aspect of the invention provides a polynucleotide encoding the beta chain portion as defined in the first aspect of the invention. As discussed above, in a particularly preferred embodiment of the invention, the alpha chain
25 portion and the beta chain portion are present on different polypeptide chains, and it is convenient (but not mandatory) that each polypeptide is encoded by a separate polynucleotide. Preferred polynucleotides encoding the alpha and beta chains are described in Figures 1 and 2, respectively. Alternatively, the two polypeptides may be encoded on the same
30 polynucleotide, in which case the two coding regions may be linked by an

(Internal Ribosome Entry Site) IRES sequence, and typically would have its own translational start and stop codons. Typically, such constructs contain two promoters, one for each TCR chain.

- 5 As discussed above, in an alternative embodiment the alpha chain portion and the beta chain portion are present in the same polypeptide, in which case a single polynucleotide may encode the single chain polypeptide.

10 In any event, the polynucleotide may be DNA or RNA, and it may or may not contain introns. Typically, the polynucleotide does not contain introns within the region that codes for the polypeptide of interest. It will be appreciated that different polynucleotides may encode the same polypeptide because of the degeneracy of the genetic code.

15 The invention also provides an expression vector that contains the polynucleotide of the invention. Such expression vectors, when present in a suitable host cell, allow for the expression of the polypeptide(s) of interest. Preferably, the expression vector is an expression vector capable of expressing a polypeptide in a mammalian cell. More preferably, the
20 expression vector is one which is able to express a polypeptide in a T cell, such as a human CTL. Typically, the expression vectors contain a promoter which is active in particular cell types, and which may be controllable (eg inducible).

25 The vector is suitably a retroviral vector which is capable of transfection into a mammalian host cell such as a human T cell. Typically, the vector is a lentiviral vector.

30 A further aspect of the invention provides a host cell comprising a polynucleotide of the invention or a vector of the invention. The host cell

may contain a polynucleotide or vector which encodes only the alpha chain portion or only the beta chain portion. However, if the host cell is to produce a TCR molecule of the invention, it contains one or more polynucleotides or vectors which encode both the alpha chain portion and
5 the beta chain portion.

The host cell may be any cell such as a bacterial cell, yeast cell, insect cell, plant cell or mammalian cell, and methods of introducing polynucleotides into such cells are well known in the art. Typically, bacterial cells, such as
10 *Escherichia coli* cells are used for general propagation and manipulation of the polynucleotides and vectors of the invention. Other host cells may be used to express the TCR molecules of the invention and, in particular, the cell may be a mammalian cell such as a human cell. As described below in relation to the therapeutic methods using the TCR molecules of the
15 invention, it is particularly desirable if the host cell is a T cell such as (and preferably) a T cell derived from a patient to be treated, typically a patient with a WT1-expressing malignancy.

Typically, a retroviral vector (or, as the case may be vectors) encoding the
20 TCR molecule of the invention is used based on its ability to infect mature human CD4⁺ or CD8⁺ T lymphocytes and to mediate gene expression: the retroviral vector system Kat is one preferred possibility (see Finer *et al* (1994) *Blood* 83, 43). High titre amphotrophic retrovirus are used to infect purified CD8⁺ T lymphocytes isolated from the peripheral blood of tumour
25 patients following a protocol published by Roberts *et al* (1994) *Blood* 84, 2878-2889, incorporated herein by reference. Anti-CD3 antibodies are used to trigger proliferation T cells, which facilitates retroviral integration and stable expression of single chain TCRs. A combination of anti-CD3 and anti-CD8 antibodies may be more effective than anti-CD3 antibodies alone.
30 Other suitable systems for introducing genes into CTL are described in

Moritz *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91, 4318-4322, incorporated herein by reference. Eshhar *et al* (1993) *Proc. Natl. Acad. Sci. USA* 90, 720-724 and Hwu *et al* (1993) *J. Exp. Med.* 178, 361-366 also describe the transfection of CTL. The commercially available Nuclofactor system, provided by AMAXA, Germany may be used to transfect T cells. Retroviral transduction of human CD8⁺ T cells is described in Stanislawski (2001) *Nat. Immunol.* 2, 962.

Methods of cloning and genetic manipulation are well known in the art and are described in detail in standard manuals such as Sambrook & Russell (2001) *Molecular Cloning*, a laboratory manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, USA.

Patients suffering from a WT1-expressing malignancy may be treated by the introduction of the TCR molecule of the invention into their own T cells (or T cells from a donor), followed by the introduction of these engineered cells into the patient. Thus, a further aspect of the invention provides a method of combating a WT-1 expressing malignancy in a patient, the method comprising introducing into the patient a T cell, preferably derived from the patient, which is modified to express the TCR molecule of the invention. Typically, (1) T cells are obtained from the patient, (2) a polynucleotide or polynucleotides encoding and capable of expressing the TCR molecule of the invention are introduced into the T cells *ex vivo* and (3) the engineered T cells are introduced into the patient. It is particularly preferred if the T cells are the patient's T cells (ie autologous).

It is particularly preferred if the patient is HLA-A2 positive.

In other words, the specificity of the T cell, preferably autologous T cell, is changed by the introduction of the TCR molecule of the invention.

5 The T cells (for example of the patient) are typically isolated from peripheral blood mononuclear cells (PBMCs), and may be CD4⁺ and CD8⁺ cells. Typically, the cells are activated using an antibody (eg an anti-CD3 or anti-CD28 antibody) so that they become receptive to transfection, for example with one or more retroviral vectors encoding the TCR molecules of the invention. The number of cells isolated, transfected and returned to the
10 patient may be determined by the physician.

Cells may be taken from a patient after a clinical response, cryopreserved, transfected and re-infused if the same patient relapses.

15 Whether or not a malignancy is one which expresses WT1 may be determined, for example using reverse transcriptase-polymerase chain reaction (RT-PCR) or using intracellular staining techniques for the WT1 protein (which may be anti-WT1 antibodies).

20 The patient is preferably a human patient although animals may be used in a research situation. It is particularly preferred that the patient is HLA-A2 positive. Whether or not a patient is HLA-A2 positive can be determined by methods well known in the art.

25 Typically, the patient is suffering from any one or more of leukaemia, breast cancer, colon cancer, lung cancer, ovarian cancer, melanoma, thyroid cancer, head and neck cancer, glioblastoma, and sarcoma.

A further aspect of the invention provides the use of a T cell, preferably a patient derived T cell, which is modified to express the TCR molecule of the invention in the manufacture of a medicament for combating a WT1-expressing malignancy in the patient.

5 As discussed above, TCR molecules in which one or more of the CDRs differ in sequence from the precise CDR sequences given in Figures 2 and 4 form part of the invention. Preferably, such TCR molecules are able to recognise the HLA-A2/RMFPNAPYL complex more effectively than a
10 TCR molecule with the precise CDR sequences. Thus, a further aspect of the invention provides a method of selecting a TCR molecule with improved binding to an HLA-A2/RMFPNAPYL complex comprising (a) providing a TCR molecule containing an alpha chain portion and a beta chain portion wherein the alpha chain portion contains three
15 complementarity determining regions (CDRs):

CDR1 α : SSYSPS

CDR2 α : YTSAATL

CDR3 α : VVSPFSGGGADGLT

20 and the beta chain portion contains three complementarity determining regions (CDRs):

CDR1 β : DFQATT

CDR2 β : SNEGSKA

CDR3 β : SARDGGEG

25 wherein at least one amino acid residue in one or more of the CDRs as given is replaced with another amino acid residue, (b) determining whether the TCR molecule binds to an HLA-A2/RMFPNAPYL complex with greater affinity than a TCR molecule without the replacement amino
30 acid(s), and (c) selecting a molecule which binds with greater affinity.

TCR molecules with altered CDRs can readily be made by protein engineering methods. For example, a TCR display library may be made in which the alpha chain and/or beta chain CDR regions are mutagenised and
5 the TCR molecules displayed using retroviral transduction on the surface of a T cell lymphoma (see Kessels *et al* (2000) *Proc. Natl. Acad. Sci. USA* 97, 14578-14583), or on the surface of a yeast or a bacteriophage. A HLA-A2/RMFPNAPYL complex may be used to select cells or bacteriophages which bind the complex with high affinity by virtue of the TCR molecule
10 that they present. TCR molecules which have a higher binding affinity (lower K_D) than a TCR molecule with the precise CDR sequences are selected for further study.

The invention will now be described in more detail by reference to the
15 following figures and non-limiting examples.

Figure 1 shows the nucleotide coding sequence of the pWT126-specific TCR-alpha chain ($V\alpha$ -1.5).

20 Figure 2 shows the protein sequence of the pWT126-specific TCR-alpha chain ($V\alpha$ -1.5). The position of the CDRs, FRs and constant region are marked. The leader sequence is shown in bold.

Figure 3 shows the nucleotide coding sequence of the pWT126-specific
25 TCR-beta chain ($V\beta$ -2.1).

Figure 4 shows the protein sequence of the pWT126-specific TCR-beta chain ($V\beta$ -2.1). The position of the CDRs, FRs and constant region are marked.

Figure 5 is a diagram showing retroviral vectors containing TCR genes. The TCR alpha and beta chains were inserted into the retroviral vector pMP71 (Engels *et al* (2003) *Human Gene Ther.* 14, 1155-1168 for gene transfer into human T cells. LTR is a long terminated repeat. PRE is posttranscriptional regulatory element.

Figure 6 is a diagram showing retroviral TCR gene transfer into human T cells. Peripheral blood lymphocytes were activated with anti-CD3 antibodies, IL-2 and IL-7, followed 3 days later by transduction with retroviral vectors encoding the WT1-specific TCR. TCR expression was monitored at day 6 using antibodies specific for the TCR-V-beta 2.1 (present in the transferred TCR). Mock transduced T cells show the percentage of un-manipulated human T cells expressing V-beta 2.1. After transduction both CD8-positive and CD8-negative (i.e. CD4-pos) T cells have an increased percentage of V-beta 2.1 cells. V-beta 2.1 DNA and amino acid sequences are shown in Figures 3 and 4.

Figure 7 shows that repeated stimulation of TCR-transduced T cells (as shown in Fig. 6) with T2 cells presenting the pWT126 peptide leads to an expansion of CD8-positive T cells expressing V-beta 2.1.

Figure 8 shows that TCR-transduced T cells (as shown in Figs. 6 and 7) stain with HLA-A2/pWT126 tetramers.

Figure 9 shows that TCR-transduced T cells (as shown in Fig. 6 and 7) kill the HLA-A2-positive T2 cells coated with the pWT126 peptide, but not T2 cells coated with the A2-binding pWT235 control peptide. The T cells also kill the HLA-A2-positive BV173 leukaemia cells expressing WT1 endogenously.

Figure 10 shows that purified TCR-transduced CD8-positive T cells kill the HLA-A2-positive T2 cells coated with the pWT126 peptide, but not T2 cells coated with the A2-binding pWT235 control peptide. The CD8-
 5 positive T cells also kill the HLA-A2-positive BV173 leukaemia cells expressing WT1 endogenously.

Figure 11 shows that a small percentage of purified CD4-positive TCR-transduced T cells stain with HLA-A2/pWT126 tetramers.

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Figure 12 shows that purified TCR-transduced CD4-positive T cells kill the HLA-A2-positive T2 cells coated with the pWT126 peptide, but not T2 cells coated with the A2-binding pWT235 control peptide. The CD4-
 15 positive T cells also kill the HLA-A2-positive BV173 leukaemia cells expressing WT1 endogenously.

Figure 13 shows that purified TCR-transduced CD8-positive T cells produce IFN- γ after stimulation with the HLA-A2-positive T2 cells coated with the pWT126 peptide, but not T2 cells coated with the A2-binding
 20 pWT235 control peptide. Also, the CD8-positive T cells produce IFN- γ after stimulation with the HLA-A2-positive BV173 leukaemia cells expressing WT1 endogenously.

Figure 14 is a schematic diagram showing the general structure of $\alpha\beta$ TCR
 25 molecules. The amino acid numbers mentioned do not necessarily correspond to those in Figures 2 and 4.

Example 1: Functionally active T cell receptor (TCR) specific for the WT-1-derived peptide pWT126 (RMFPNAPYL)

We have cloned a T cell receptor (TCR) that is specific for a peptide (pWT126; RMFPNAPYL) of the Wilms Tumour antigen-1 (WT1) presented by HLA-A2 class I molecules. The WT1 transcription factor is expressed in various human malignancies, including leukaemia, breast cancer, colon cancer, lung cancer, ovarian cancer and others. The CTL from which the TCR was cloned show killing activity against human cancer cells that express WT1, but not against normal human cells that express physiological levels of WT1.

The therapeutic goal is to equip patient T cells with this potent and specific killing activity by transfer of the genes encoding the TCR. For this, we have inserted the TCR genes into retroviral vectors and demonstrated that gene transduced human T cells show killing activity against WT1 expressing human cancer and leukemia cell lines. The specificity profile of this CTL line has been described in several research papers and can be summarized as: (1) Killing of HLA-A2-positive targets coated with the WT1-derived peptide pWT126 (Gao *et al* (2000) *Blood* 95, 2198-2203); (2) Killing of fresh HLA-A2-positive leukaemia cells expressing WT1 (Gao *et al* (2000) *Blood* 95, 2198-2203); (3) Killing of HLA-A2-positive leukemia CFU progenitor cells (Gao *et al* (2000) *Blood* 95, 2198-2203; Bellantuono *et al* (2002) 100, 3835-3837); (4) Killing of HLA-A2-positive leukaemia LTC-IC stem cells (Bellantuono *et al* (2002) *Blood* 100, 3835-3837); (5) Killing of HLA-A2-positive NOD/SCID leukaemia initiating cells (Gao *et al* (2003) *Transplantation* 75, 1429-1436); and (6) No killing of normal HLA-A2-positive NOD/SCID engrafting hematopoietic stem cells (Gao *et al* (2003) *Transplantation* 75, 1429-1436). We have now shown that human T cells transduced with the WT1-specific TCR display similar specificity as the CTL line from which the TCR was cloned.

The data described in detail in the legends to Figures 1 to 13 indicate that TCR gene transfer into human T cells is feasible and that it leads to the surface expression of the introduced TCR chains. The recipient T cells show killing activity against HLA-A2-positive targets coated with the pWT126 peptide. The TCR-transduced T cells also kill human tumour cells expressing WT1 endogenously. In addition, the transduced T cells produce IFN-g in an HLA-A2-restricted, peptide-specific fashion. Finally, the transferred TCR can function in CD4-positive helper T cells. These CD4-positive T cells show HLA-A2-restricted, antigen-specific killing activity and antigen-specific cytokine production (not shown). This indicates that TCR gene transfer can be used to confer HLA class I-restricted antigen-specific effector function to both CD8-positive and CD4-positive human T cells.

15 **Example 2: Selection and treatment of a patient**

Peripheral blood monocyte cells (PBMCs) are taken from an HLA-A2-positive patient who has a WT1-expressing malignancy. The PBMCs are activated with anti-CD3/CD28 antibodies added to the culture or on beads for 3 days and then transduced with TCR encoding retroviral particles as described in Example 1. At day 5 we can demonstrate that transduced CD4 and CD8 T cells express the introduced TCR. At day 6 we can demonstrate antigen-specific activity of the transduced T cells. At day 6 the transduced T cells are reinfused into the patient.

CLAIMS

1. A T cell receptor molecule (TCR) containing an alpha chain portion and a beta chain portion wherein the alpha chain portion contains three complementarity determining regions (CDRs):

CDR1 α : SSYSPS

CDR2 α : YTSAATL

CDR3 α : VVSPFSGGGADGLT

and the beta chain portion contains three complementarity determining regions (CDRs):

CDR1 β : DFQATT

CDR2 β : SNEGSKA

CDR3 β : SARDGGEG, or wherein up to three amino acid residues in one or more of the CDRs are replaced by another amino acid residue.

2. A TCR molecule according to Claim 1 wherein the alpha chain portion and the beta chain portion are present on different polypeptide chains.

3. A TCR molecule according to Claim 1 wherein the alpha chain portion and the beta chain portion are present in the same polypeptide chain.

4. A TCR molecule according to any of Claims 1 to 3 wherein the CDRs are grafted to a human framework region.

5. A TCR molecule according to Claim 4 wherein the alpha chain portion has the amino acid sequence given in Figure 2.

6. A TCR molecule according to Claims 4 or 5 wherein the beta chain portion has the amino acid sequence given in Figure 4.
7. A TCR molecule according to any one of Claims 1 to 6 which is soluble.
8. A polynucleotide encoding the alpha chain portion as defined in Claim 1.
9. A polynucleotide encoding the beta chain portion as defined in Claim 1.
10. A polynucleotide encoding the single chain TCR molecule as defined in Claim 3.
11. An expression vector comprising a polynucleotide according to any of Claims 8 to 10.
12. An expression vector according to Claim 11 which is a retroviral vector.
13. A host cell comprising a polynucleotide according to any of Claims 8 to 10 or an expression vector according to Claims 11 or 12.
14. A host cell according to Claim 13 which is a T cell.
15. A host cell according to Claim 14 which is a T cell derived from a patient.

16. A method of combating a WT1-expressing malignancy in a patient, the method comprising introducing into the patient a T cell, preferably derived from the patient, which is modified to express the TCR molecule of any of Claims 1 to 7.

5

17. A method according to Claim 16 comprising (1) obtaining T cells from the patient, (2) introducing into the T cells a polynucleotide according to any of Claims 8 to 10 or an expression vector according to Claims 11 or 12 so that the T cell expresses the encoded TCR molecule and (3) introducing the cells from step (2) into the patient.

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18. A method according to Claim 16 or 17 wherein the WT1-malignancy is any one or more of breast cancer, colon cancer, lung cancer, leukaemia, ovarian cancer, melanoma, head and neck cancer, thyroid cancer, glioblastoma and sarcoma.

15

19. Use of a T cell, preferably a patient derived T cell, modified to express the TCR molecule of any of Claims 1 to 7 in the manufacture of a medicament for combating a WT1-expressing malignancy in the patient.

20

20. Use according to Claim 19 wherein a polynucleotide according to any of Claims 8 to 10 or an expression vector according to Claims 11 or 12 has been introduced into the T cell, preferably patient derived T cell, so that the T cell expresses the encoded TCR molecule.

25

21. A method of selecting a TCR molecule with improved binding to an HLA-A2/RMFPNAPYL complex comprising (a) providing a TCR molecule containing an alpha chain portion and a beta chain portion wherein the alpha chain portion contains three complementarity

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determining regions (CDRs):

CDR1 α : SSYSPS

CDR2 α : YTSAATL

CDR3 α : VVSPFSGGGADGLT

5 and the beta chain portion contains three complementarity
determining regions (CDRs):

CDR1 β : DFQATT

CDR2 β : SNEGSKA

CDR3 β : SARDGGEG.

10

wherein at least one amino acid residue in one or more of the CDRs
as given is replaced with another amino acid residue, (b) determining
whether the TCR molecule binds to an HLA-A2/RFMPNAPYL
complex with greater affinity than a TCR molecule without the
15 replacement amino acid(s), and (c) selecting a molecule which binds
with greater affinity.

22. Any novel method of combating cancer as herein described.

ABSTRACT

A T cell receptor molecule (TCR) containing an alpha chain portion and a beta chain portion wherein the alpha chain portion contains three complementarity determining regions (CDRs):

CDR1 α : SSYSPS

CDR2 α : YTSAATL

CDR3 α : VVSPFSGGGADGLT

and the beta chain portion contains three complementarity determining regions (CDRs):

CDR1 β : DFQATT

CDR2 β : SNEGSKA

CDR3 β : SARDGGEG, or wherein up to three amino acid residues in one or more CDRs are replaced by another amino acid residue.

The invention also includes polynucleotides encoding the TCR molecules, and host cells containing the said polynucleotides. Patient derived T cells may have the polynucleotides encoding the TCR molecules introduced therein, and the engineered T cells may be introduced into the patient in order to combat a WT1-expressing malignancy.

Figure 2.

Figure 1:

Human TCR V α -1.5 (V α -8.2) coding sequence

ATGCTCCTGC TGCTCGTCCC AGTGCTCGAG GTGATTTTAA CTCTGGGAGG
AACCAGAGCC CAGTCGGTGA CCCAGCTTGA CAGCCACGTC TCTGTCTCTG
AAGGAACCCC GGTGCTGCTG AGGTGCAACT ACTCATCTTC TTATTCACCA
TCTCTCTTCT GGTATGTGCA ACACCCCAAC AAAGGACTCC AGCTTCTCCT
GAAGTACACA TCAGCGGCCA CCCTGGTTAA AGGCATCAAC GGTTTTGAGG
CTGAATTTAA GAAGAGTGAA ACCTCCTTCC ACCTGACGAA ACCCTCAGCC
CATATGAGCG ACGCGGCTGA GTACTTCTGT GTGTGAGTC CTTTTCAGG
AGGAGGTGCT GACGGACTCA CCTTTGGCAA AGGACTCAT CTAATCATCC
AGCCCTATAT CCAGAACCCCT GACCCTGCCG TGTACCAGCT GAGAGACTCT
AAATCCAGTG ACAAGTCTGT CTGCCTATTC ACCGATTTTG ATTCTCAAAC
AAATGTGTCA CAAAGTAAGG ATTCTGATGT GTATATCACA GACAAAAC TG
TGCTAGACAT GAGGTCTATG GACTTCAAGA GCAACAGTGC TGTGGCCTGG
AGCAACAAAT CTGACTTTGC ATGTGCAAAC GCCTTCAACA ACAGCATTTAT
TCCAGAAGAC ACCTTCTTCC CCAGCCCAGA AAGTTCCTGT GATGTCAAGC
TGGTCGAGAA AAGCTTTTGA ACAGATACGA ACCTAAACTT TCAAAACCTG
TCAGTGATTG GGTTCCGAAT CCTCCTCCTG AAAGTGGCCG GGTTTAAATCT
GCTCATGACG CTGCGGCTGT GGTCCAGCTG A

Figure 2

Human TCR V α -1.5 (V α -8.2) protein sequence

		FR1
MLLLLVPVLEVIFTLGGTRAQSVTQLDSHVS		SEGT
	CDR1	FR2
PVLLRCNYSSSY	<u>SPSLFWYVQHPNKGLQLLLKYT</u>	
	CDR2	FR3
<u>SAAATLVKGIN</u>	<u>GFEAEFKKSETSFHLTKPSAHMSDA</u>	
	CDR3	
<u>AEYFCVVSPESGGGADGLT</u>		
constant		
FGKGT	HLIQPYIQNP	DPAVYQLRDSKSSDKSVCLF
TD	FDSQTNVS	QSKDSDVYITDKTVLDMRSM
DFK	SNSAVAWSNKSDFACAN	AFNNSIIPED
TF	FPS	ESSCDVKLVEKSFETDTNLNFQNL
LL	KVAGFNLLMT	LRLWSS

Figure 3:

Human TCR V β -2.1 (V β -20.1) coding sequence

ATGCTGCTGC TTCTGCTGCT TCTGGGGCCA GGCTCCGGGC TTGGTCTGT
CGTCTCTCAA CATCCGAGCT GGGTTATCTG TAAGAGTGGA ACCTCTGTGA
AGATCGAGTG CCGTTCCCTG GACTTTCAGG CCACAACATAT GTTTTGGTAT
CGTCAGTTCC CGAAACAGAG TCTCATGCTG ATGGCAACTT CCAATGAGGG
CTCCAAGGCC ACATACGAGC AAGCGGTCGA GAAGGACAAG TTTCTCATCA
ACCATGCAAG CCTGACCCTTG TCCACTCTGA CAGTGACCAG TGCCCATCCT
GAAGACAGCA GCTTCTACAT CTGCAGTGCT AGAGATGGGG GGGAGGGTTC
GGAGACCCAG TACTTCGGGC CAGGCACGCG GCTCCTGGTG CTCGAGGACC
TGAAAAACGT GTTCCACCC GAGGTCGCTG TGTTCAGCC ATCAGAAGCA
GAGATCTCCC ACACCCAAA GGCCACACTG GTGTGCCCTGG CCACAGGCTT
CTACCCCGAC CACGTGGAGC TGAGCTGGTG GGTGAATGGG AAGGAGGTGC
ACAGTGGGT CAGCACAGAC CCGCAGCCCC TCAAGGAGCA GCCCGCCCTC
AATGACTCCA GATACTGCCCT GAGCAGCCGC CTGAGGGTCT CGGCCACCTT
CTGGCAGAAC CCCCAGCAACC ACTTCGCTG TCAAGTCCAG TTCTACGGGC
TCTCGGAGAA TGACGAGTGG ACCCAGGATA GGGCCAAACC TGTACCCAG
ATCGTCAGCG CCGAGGCCCTG GGGTAGAGCA GACTGTGGCT TCACCTCCGA
GTCTTACCAG CAAGGGGTCC TGTCTGCCAC CATCCTCTAT GAGATCTTGC
TAGGGAAGGC CACCTTGAT GCCGTGCTGG TCAGTGCCCT CGTGCTGATG
GCCATGGTCA AGAGAAAGGA TTCCAGAGGC TAG

Figure 4

Human TCR V β -2.1 (V β -20.1) protein sequence

FR1
MLLLLLLLGPGSGLGAVVSQHPSWVICKSGTTSVKIECR

CDR1 FR2 CDR2
SLDFQATTMFWRQFPKQSLMLMATSNEGSKATYEQ

FR3
GVEKDKFLINHASLTLSLTLTVTSAHPESSFYICSARD

CDR3
GGEG

constant
SETQYFGPGRLLVLEDLKNVFPPEVAVFEPSEAEISHTQ
KATLVCLATGFYPDHVELSWWVNGKEVHSGVSTDPQPL
KEQPALNDSRYCLSSRLRVSA¹TFWQNP¹RNH¹ERCQVQFY
GLSENDEWTQDRAKPV¹TQIVSAEA¹WGRADCGFTSESYQ
QGVLSATILYEILLGKATLYAVLV¹SALVLMAMV¹KRKDS
RG

Figure 5:

TCR-retroviral constructs

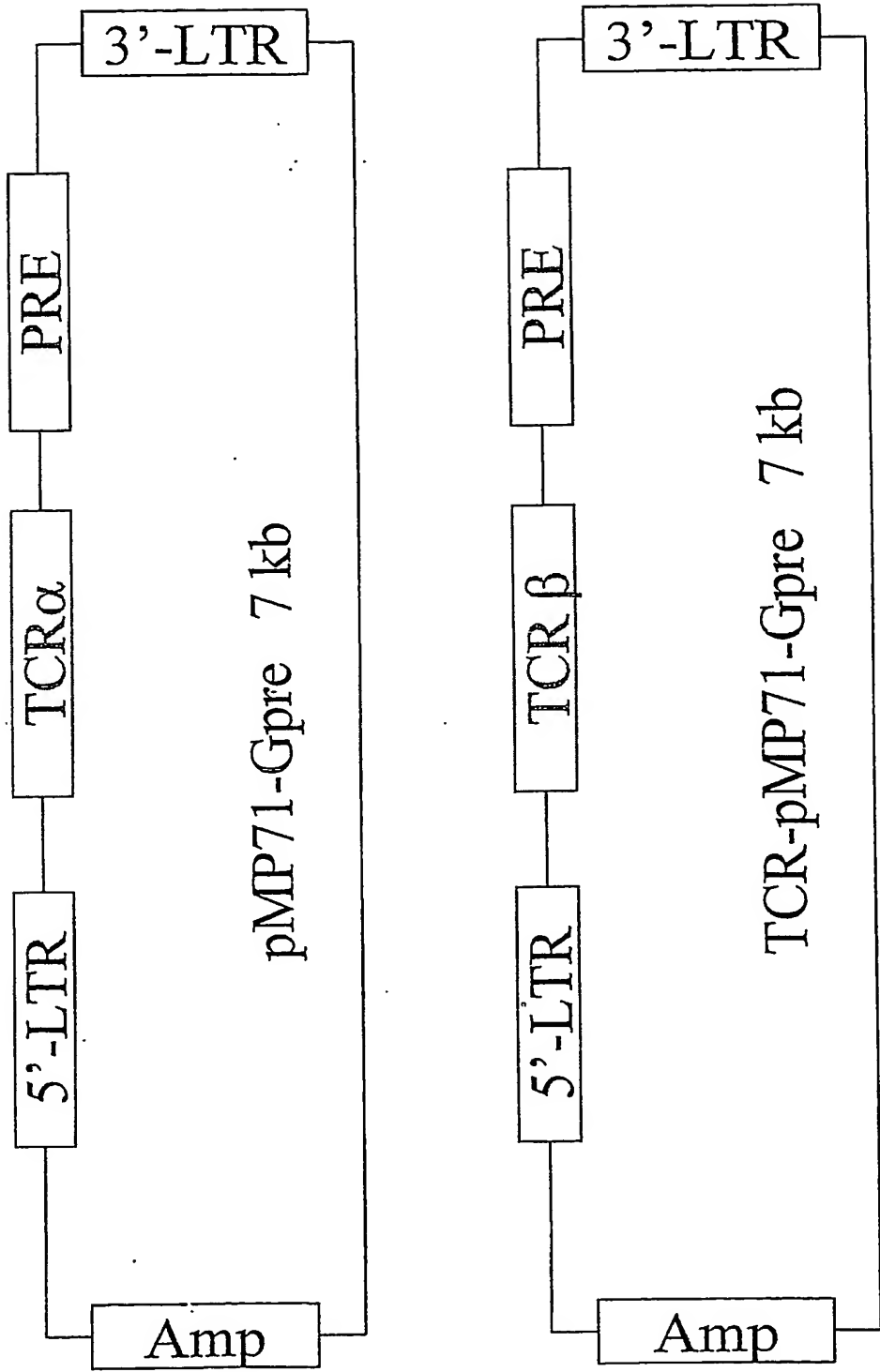


Figure 6:

TCR Expression in Human PBMC after transduction

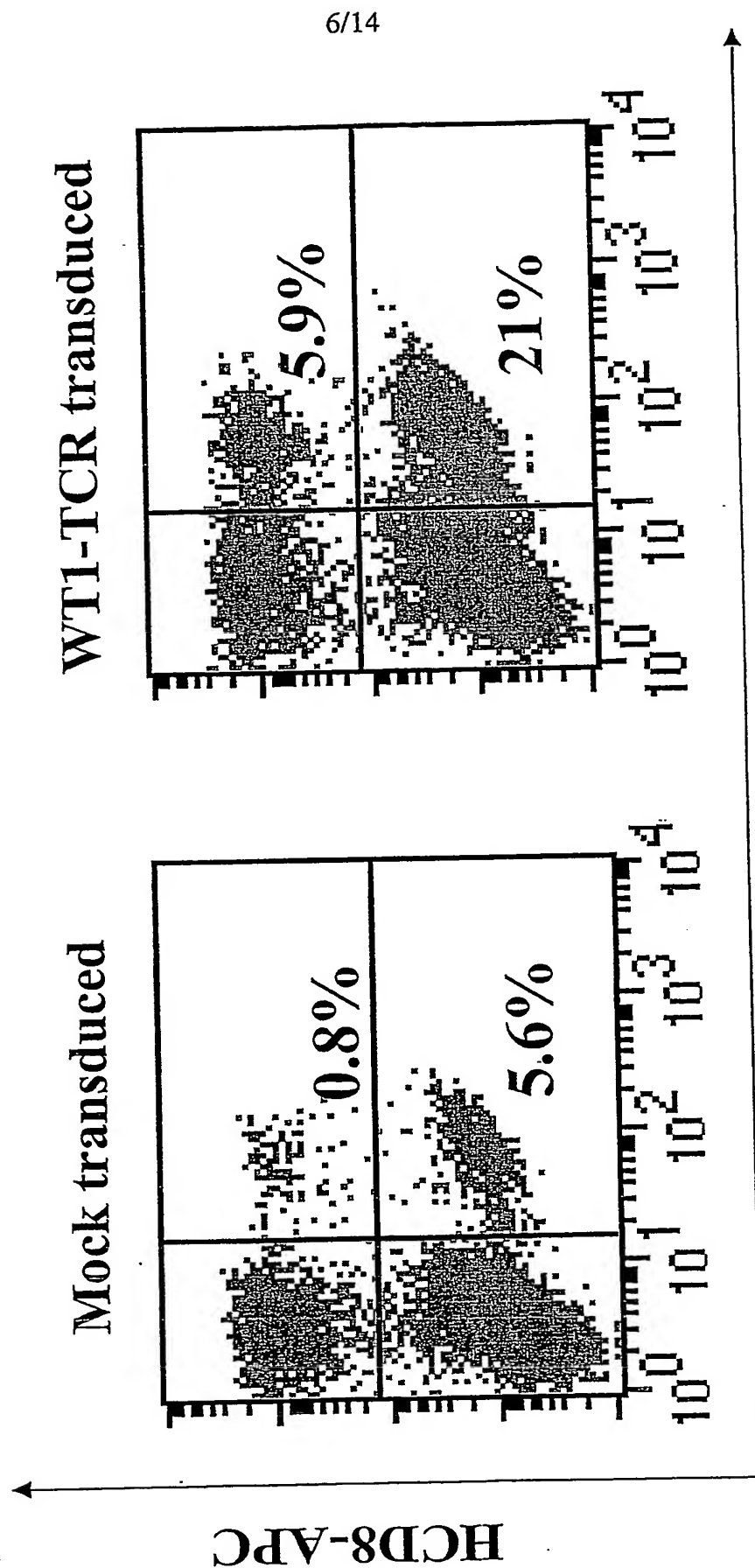


Figure 7:

**Increase of CD8⁺-Vb2.1⁺ T Cells
after antigen stimulation**

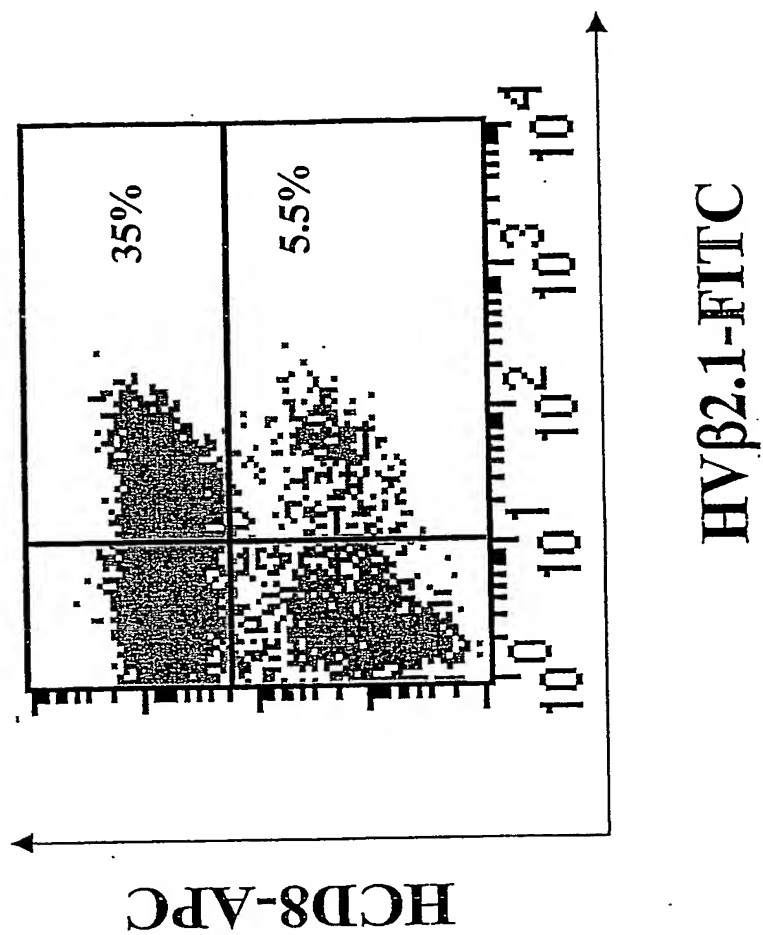


Figure 8:

TCR specific for pWT126 transduced PBMC

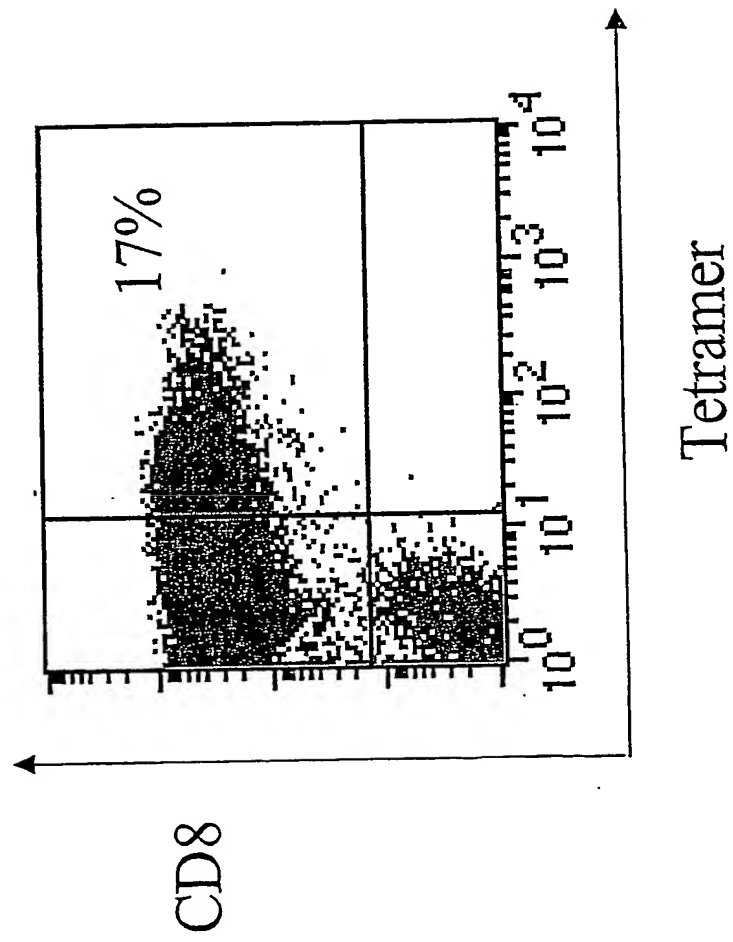


Figure 9:

TCR transduced bulk T cells show pWT126-specific killing activity

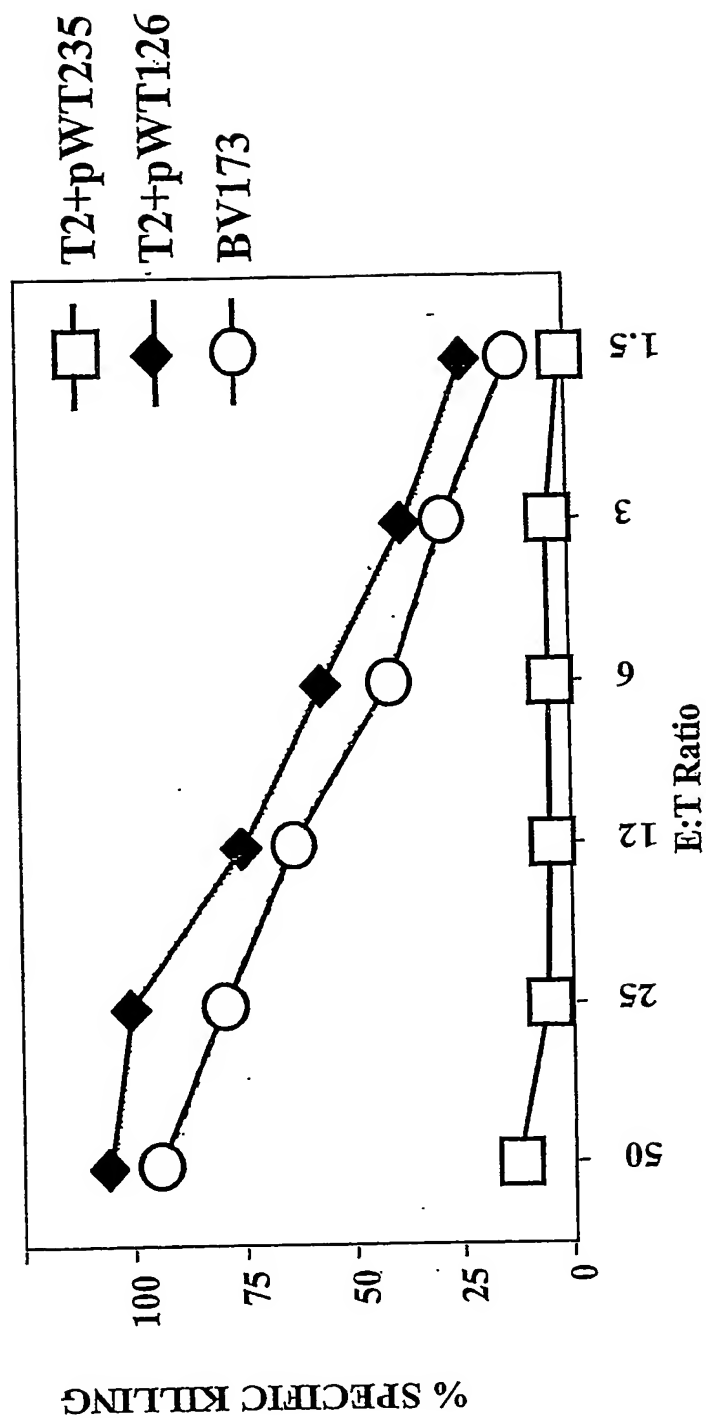


Figure 10:

TCR transduced CD8⁺ T cells show pWT126-specific killing activity

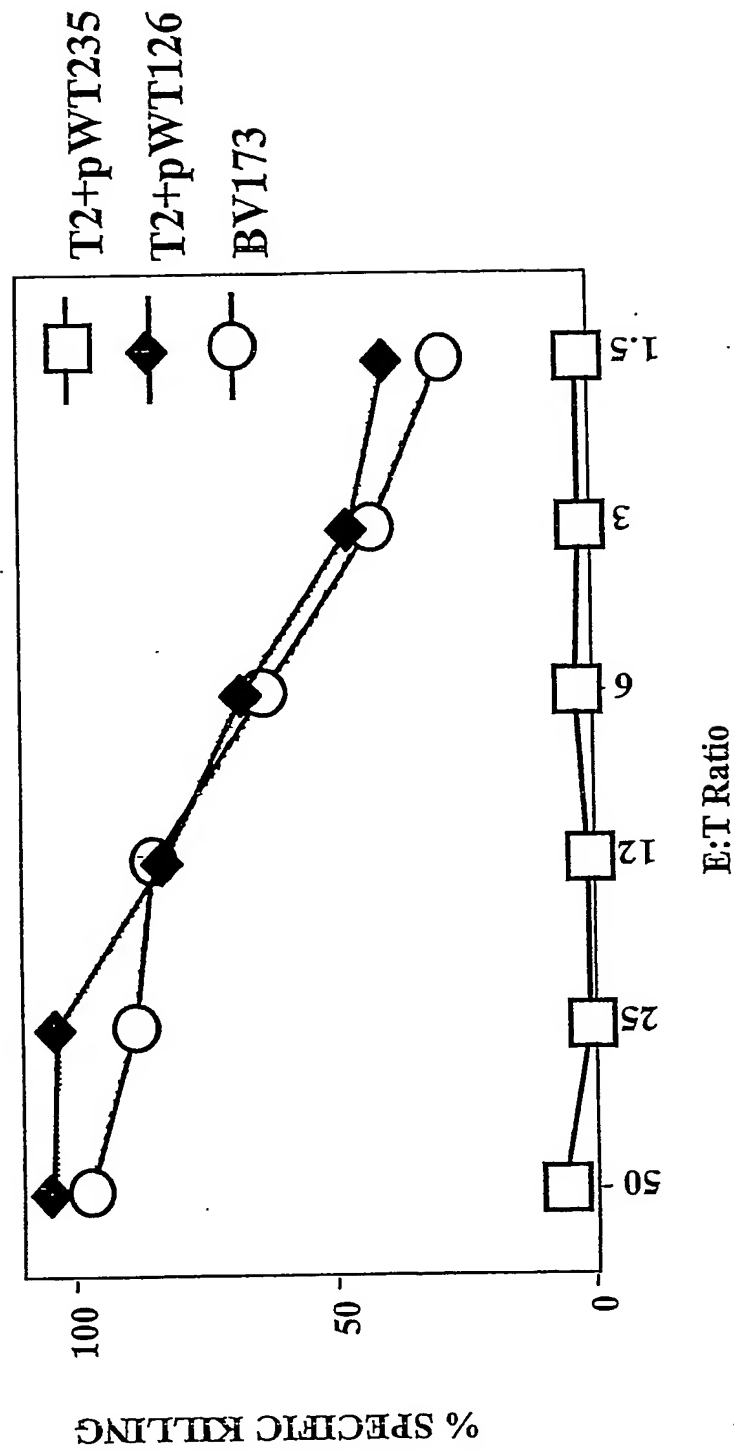


Figure 11:

TCR specific for pWT126 transduced
PBMC sorted CD4

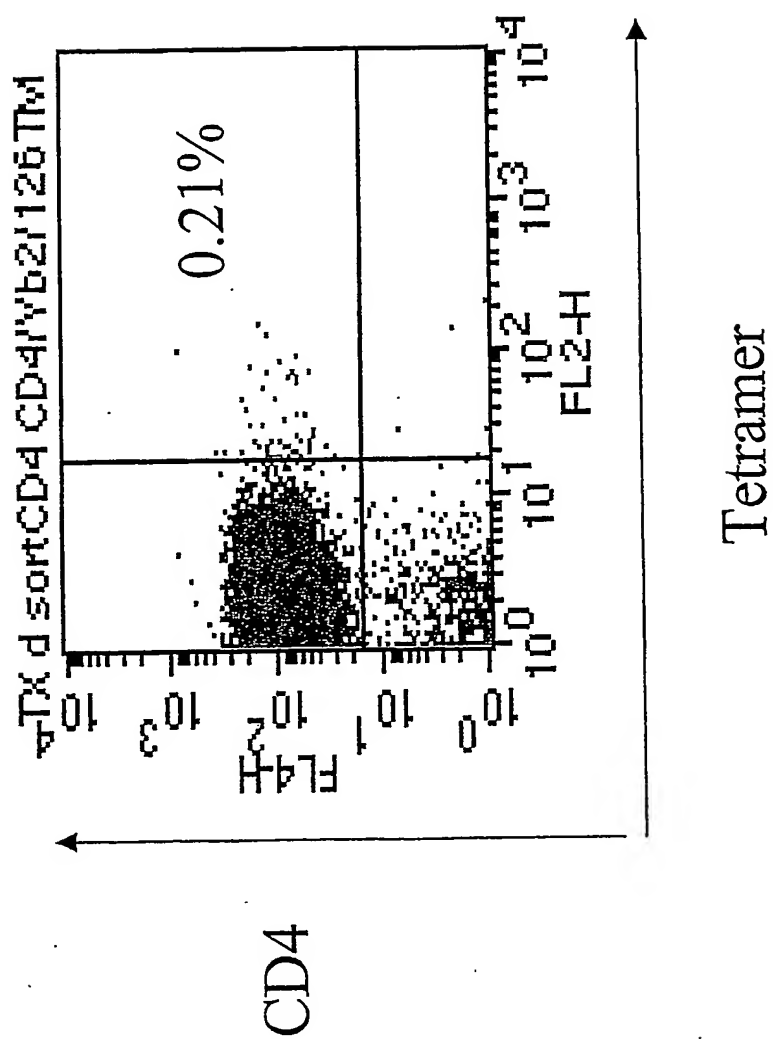


Figure 12:

TCR transduced CD4+ T cells show pWT126-specific killing activity

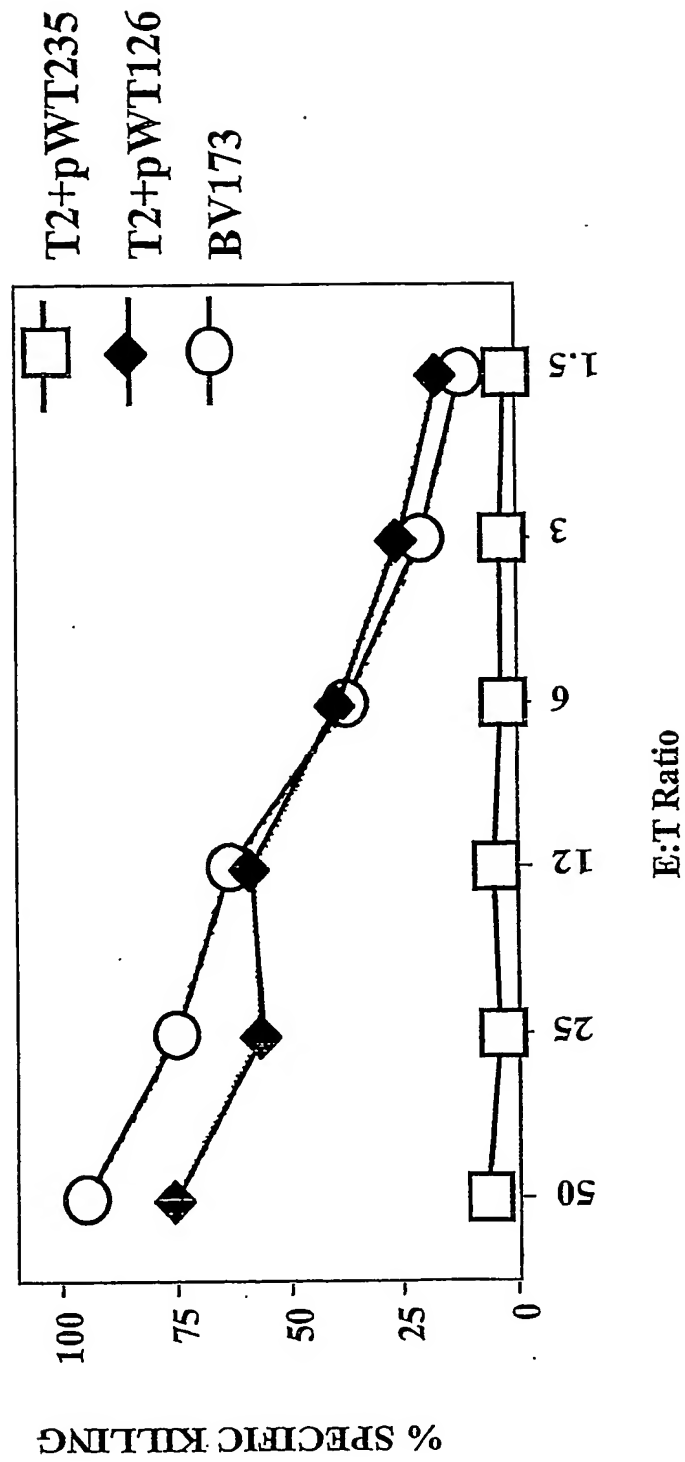
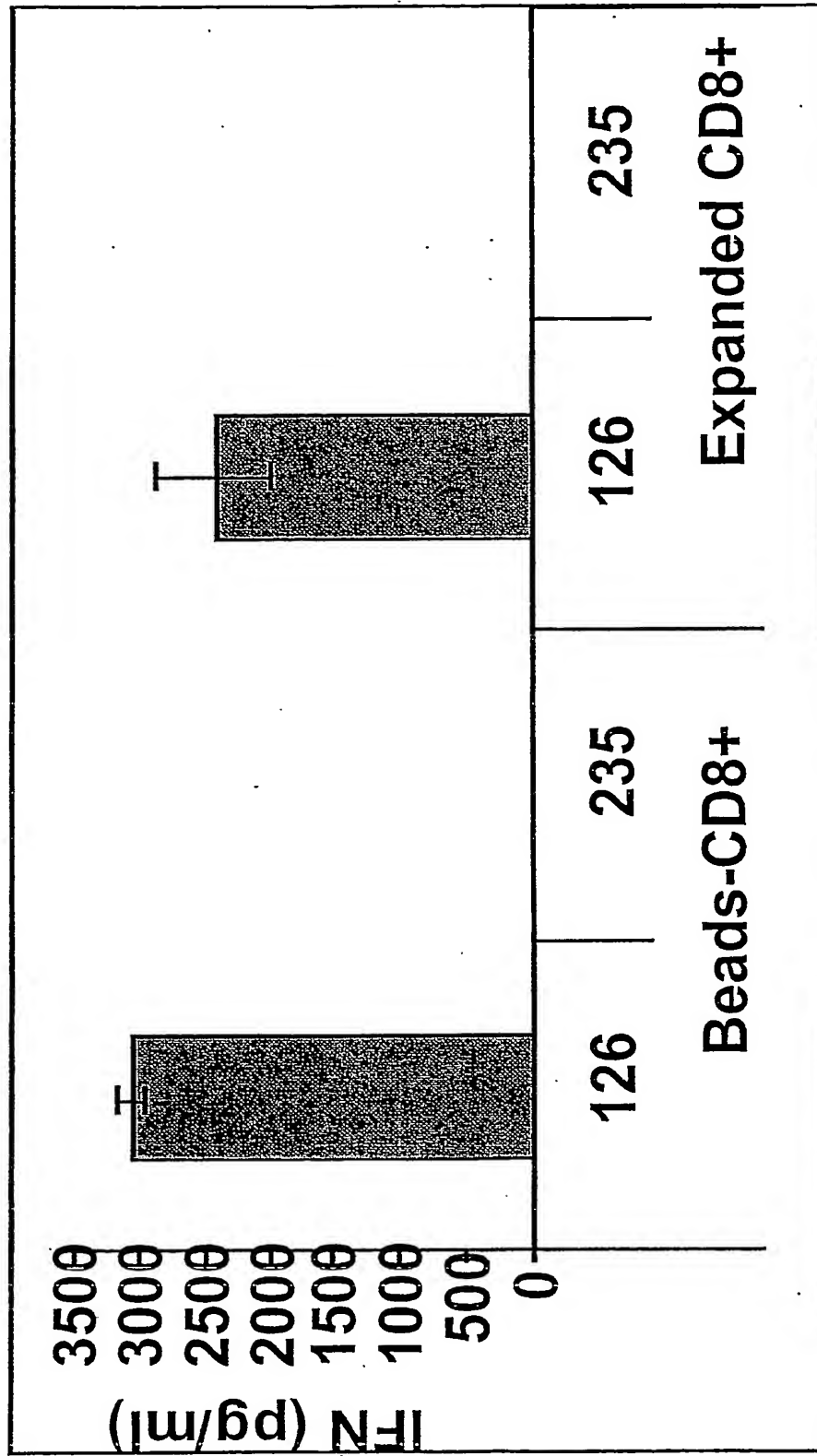


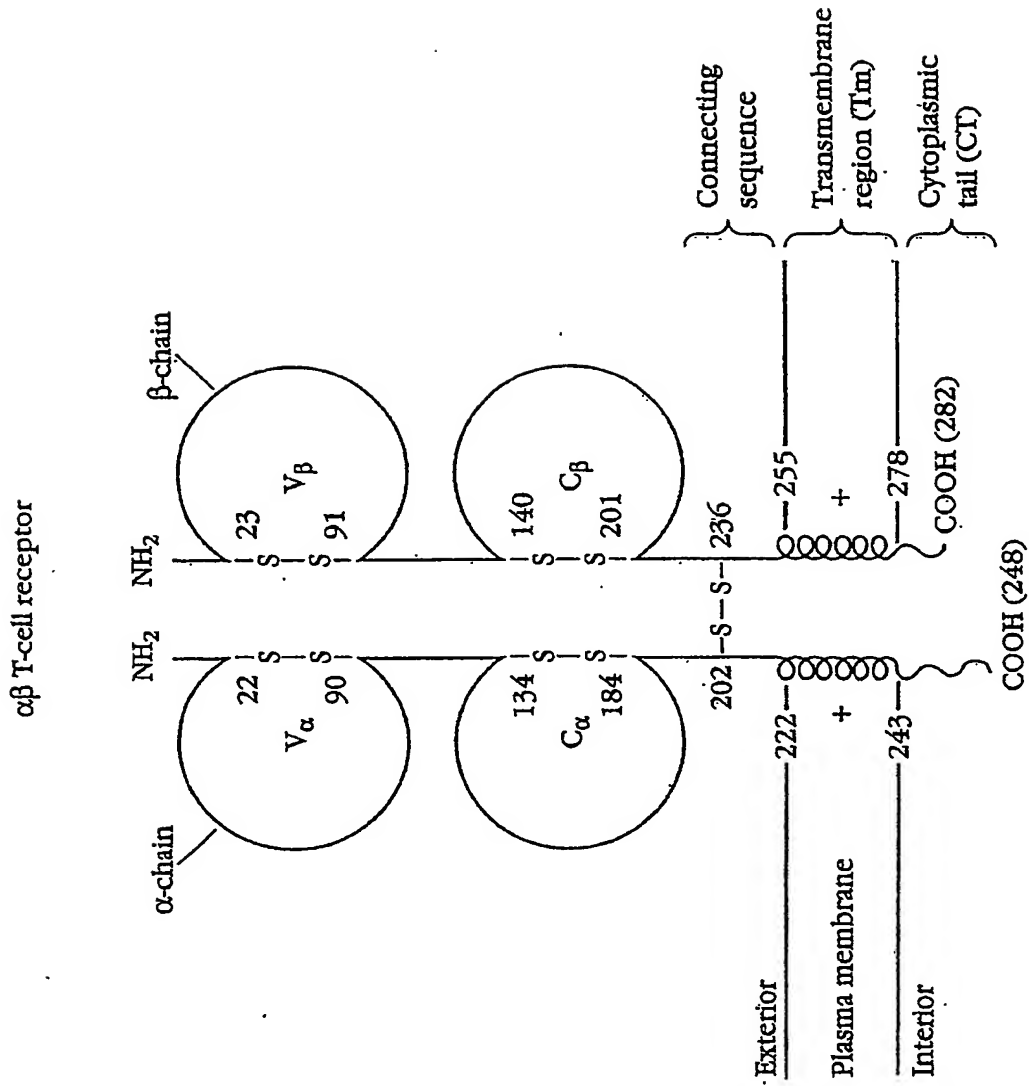
Figure 13:

TCR transduced CD8⁺ T cells show pWT126-specific IFN- γ production



After 20 hrs incubation

Figure 14



Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/GB04/005100

International filing date: 06 December 2004 (06.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: GB
Number: 0328363.7
Filing date: 06 December 2003 (06.12.2003)

Date of receipt at the International Bureau: 24 January 2005 (24.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



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